

TRANSGENIC TEA THROUGH BIOLISTIC USING LEAF EXPLANTS

Field of the invention:

The present invention relates to Production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through biolistic

Background of the invention:

Tea is a popular caffeine containing beverage with anti-cancerous properties (Jankun, J., Selman, S.H., Swiercz, R. Why drinking green tea could prevent cancer. Nature 5:561; 1997). Tea is also an important employment generator and a major foreign exchange earner in all the tea growing areas of the world (Wilson, K.C. Botany and Plant Improvement In: Wilson R.C., ed. Coffea, Cocoa and Tea. CABI Publishing, Wallingford, UK: 167-173; 1999). While, the total production of tea is not sufficient enough to meet the demands of the domestic and the world markets (Kabra, G.D. Tea statistics for 1999 In: Tea time, Vol. VIII, No. 3 Sep-Nov 99, 30-31; 1999). The yield and quality of tea is further reduced by different biotic (fungi, pests and viruses) and abiotic (frost, hail, chilling, drought, nutritional deficiencies etc.) stresses (Wilson, K.C. Botany and Plant Improvement In: Wilson R.C., ed. Coffea, Cocoa and Tea. CABI Publishing, Wallingford, UK: 167-173; 1999). Although for most crops, higher yield per unit area is of primary importance but the major objective for tea is improved yield coupled with better adaptability and cup characters. Moreover, the world market has critical standards for tea from different parts of the world to which the products must conform in order to attain high commercial value.

Superior stress resistant tea plants combining both yield and cup quality are therefore, of utmost importance (Barua, D.N. The tea plant of commerce In: Barua, D.N., ed. Science and practice in tea culture, Tea Research Association Calcutta; 53-68; 1989). Crop improvement programmes also aim at reasonable degree of morphological homogeneity in the progeny. Long life cycles of almost 10 years coupled with high degree of self incompatibility and inbreeding depression (Barua, D.N. The tea plant of commerce In: Barua, D.N., ed. Science and practice in tea culture, Tea Research Association Calcutta; 53-68; 1989) are the major limitations for conventional tea

breeding programmes. The important and efficient alternative for overcoming these limitations is genetic transformation through *Agrobacterium tumefaciens* or biolistic wherein desired genes can be directly introduced into the plant genome.

Biolistic has been successfully employed in the genetic improvement of woody perennials specially when the plants have long life cycle or when the basic information about plant inheritance is lacking. Thus genetic transformation through biolistic holds a tremendous potential in tea specially when the leaves are used as the initial explants. More so, because leaf explants despite having a tremendous potential for crop improvement are highly recalcitrant to *Agrobacterium tumefaciens*-mediated transformation probably due to the presence of certain phenolics (Biao Xi, Toru K, Jian Xu, Yongyan B Effect of polyphenol compounds in tea transformations. Abstr. no. 314. In: American Society of Plant Physiologists, Plant Biology 1998).

Although some tea clones have been identified which are high yielding as well as of high quality, yet these are susceptible to blister blight disease. Biotechnological improvement through homogenous tissues like leaf explants is required in these clones because heterogeneous tissues like cotyledon explants would result in genetic variability and loss of the desirable character of high yield and good quality. Therefore, use of leaf explants was important. However, the transformation of leaves through *Agrobacterium tumefaciens* is known to be ineffective due to high content of certain polyphenols.

It was realized that primarily three factors viz. (i) increased surface area for maximum particle penetration, (ii) minimum cell damage/injury and (iii) maximum regeneration efficiency were required in order to make the transgenic protocol successful. Therefore, a method for biolistic mediated transgenic production of tea (*Camellia sinensis* (L.) O. Kuntze) using leaf explants was developed taking into account the above three factors in order to enable further genetic improvement of selected elites.

Genetic transformation through *Agrobacterium tumefaciens* was first initiated in tea leaves (Matsumoto S and Fukai M 1998 *Agrobacterium tumefaciens* mediated gene transfer in tea plant (*Camellia sinensis*) cells. Japan Agricultural Research Quarterly, 32: 287-291; Matsumoto S and Fukai M 1999 Effect of acetosyringone application on *Agrobacterium* mediated gene transfer in tea plant (*Camellia sinensis*), Bulletin of the National Research Institute of vegetables, ornamental plants and tea, Shizuoka, Japan, 14:

9-15) wherein transformed leaf callus was produced using 500 μ M Acetosyringone was selected at 200 μ g/ml kanamycin. These transformed calli showed PCR amplification for nptII gene primers. The major draw back is that transgenic plants could not be regenerated from these transformed leaf calli. Even induction of callus on the leaves required a very high dose of the costly chemical Acetosyringone.

Genetic transformation through *Agrobacterium tumefaciens* has also been attempted by Biao (Biao Xi, Toru K, Jian Xu, Yongyan B Effect of polyphenol compounds in tea transformations. Abstr. no. 314. In: American Society of Plant Physiologists, Plant Biology 1998) wherein leaf and cotyledons were tested. The draw back of the report is that the leaf explants could not be significantly infected with *Agrobacterium tumefaciens* and could not be transformed because of high content of phenolics mainly catechins.

Objects of the invention:

The main object of the present invention is to provide a method for production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through biolistic using leaf explants which obviates the drawbacks as detailed above. The novelty of this method is that it is the first successful method of transforming leaf explants of tea in high frequency with the use of biolistics so as to produce transgenic plants.

Another object of the present invention is the achievement of a combination of increased surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency.

Another object of the present invention is to develop different combinations (354) of the parameters that affect biolistic in order to achieve (i) increased surface area for maximum particle penetration, (ii) minimum cell damage/injury and (iii) maximum regeneration efficiency.

Another object of the present invention is to overcome some of the problems faced in certain steps during biolistics.

Another object of the present invention is the production of transgenic tea resistant to biotic and abiotic stresses.

Yet another object of the present invention is to produce tea plants with higher yield and good cup quality.

Yet another object of the present invention is to genetically transform elite tea plants so as to improve both quality and yield.

Yet another object of the present invention is to produce de-caFFEinated tea plants.

Yet another object of the present invention is to produce transgenic tea plants with sweet tea leaves using genes like thaumatin and lectins etc.

Detailed description of the invention:

The invention relates to production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through novel combination of 360 parameters for the production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through biolistic. The method of the invention comprises

- (a) prior to the subjection of the leaf explants to 360 combinations, treatment of leaf explants with different concentrations (0.25-0.75M) of different osmotic agents ranging from sucrose, myoinositol, sorbitol, mannitol alone, combinations of mannitol and sorbitol and liquid basal MS medium (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497; 1962) supplemented with vitamins like thiamine-HCl (0.1 mg/l), pyridoxine-HCl (0.5 mg/l) and nicotinic acid (0.5 mg/l) together with glycine (2.0 mg/l) for different time periods ranging from 2 to 8 hrs
- (b) drawing of concentric circles of variable diameter ranging from 2.0-9.0 cm, on a transparent polythene sheet wherein the diameter of the outermost circle is same as that of a 9.0 cm petridish
- (c) arrangement of leaf explants with the adaxial surface up on the regeneration medium for bombardment
- (d) arrangement of leaf explants on the regeneration medium within the different concentric circles (2.0 to 5.0 cm) of 9.0 cm Petri-dishes for maximum spread of pRT99GUS plasmid DNA coated micro-projectiles produced by BioRad
- (e) sterilization of the gold particles by washing with 70% alcohol followed by sterile water for three times each
- (f) suspension of 60µg of gold particles in 0.5-1.5 ml sterile distilled water

- (g) dispersion of 25-60 μ l of this suspension in 1.5 ml Eppendorf tubes for each bombardment
- (h) mixing of 50 μ l of gold suspension with 10 μ l of different concentrations of pRT99GUS plasmid DNA (0.5-5 μ g/ μ l), 40-50 μ l of 1.5-3.5M CaCl_2 and 10-50 μ l of 0.5-2.0M spermidine free of phosphate salts with simultaneous vortexing from time to time, spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant and washing with 70% ethanol and final suspension in 50-100 μ l of 100% ethanol
- (i) coating of 10 μ l suspension of gold particles and DNA on sterile macro-carriers (BioRad) with immediate vortexing
- (j) development of 360 combinations comprising of: gap distances or distance between the rupture disc and the macro-carrier (1/4-3/8 inches alone and in combination), macrocarrier flight distance or the distance between macrocarrier and stopping screen (6-16 mm), and target distance or distance between the microprojectile stopping screen and target tissue (6-12 cm), for increasing the surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency
- (k) bombardment of leaf explants with biolistic guns like DuPont, Gene booster but specifically Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 22 to 28 inches mercury with gold particles (0.6-1.6 μ m), together with 1, 2 and 4 μ g/ μ l concentrations of DNA and the above 360 combinations wherein the tissue damage due to gas shock and high particle dispersion was circumvented by increasing the target distance for optimal particle dispersion and simultaneously the tissue damage due to off centred flight of microprojectile flight distance was overcome by decreasing the gap distance
- (l) bombardment of each plate twice after changing the direction of the Petri-plate by turning it by 180°
- (m) turning of the bombarded explants ranging from leaf, somatic embryos, zygotic embryos and embryogenic calli upside down on the regeneration medium with

abaxial surface up such that the bombarded surface touches the regeneration medium

- (n) culturing in dark for two days at a temperature of $25 \pm 2^\circ\text{C}$ of culture lab
- (o) assay of the bombarded leaves for GUS expression following the method of Jefferson, wherein the reaction of the GUS (5-bromo, 4-chloro, 3-indolyl, β -D-glucuronide) chemical with the transformed leaf explant produced a blue colour 'reaction product' thereby indicating the entry of the pRT99GUS plasmid DNA coated microprojectiles of BioRad into the cells of the explant tissue (Jefferson RA 1987, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405)
- (p) transfer of the bombarded leaf explants after two days to the regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001 under normal photoperiods of 16 h under cool fluorescent light of $52 \mu\text{mol m}^{-2}\text{s}^{-1}$ of the culture lab
- (q) finally selection of putative transformants after every 15 days on selection medium containing kanamycin (250-1100 $\mu\text{g/ml}$) (r) regeneration of shoot buds from the completely folded, half opened or fully expanded leaf explants of 3 to 5 months old *in vitro* raised cultures following the protocol of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001 (s) growing and multiplying the transgenic shoots in liquid medium of Sandal I, Bhattacharya A, Ahuja P.S. An efficient liquid culture system for tea shoot proliferation Plant Cell Tissue Organ Culture 65(1): 75-80 (2001) (t) molecular characterization of GUS positive tissues of transgenic plants selected on 250-1100 $\mu\text{g/ml}$ kanamycin using PCR and Southern Hybridization following standard methods.

In an embodiment, different explants like leaf, somatic embryos, zygotic embryos and embryogenic calli of different cultivars (*Chinary*, *Cambod* and *Assamica*) were genetically transformed through biolistics as stated above.

In another embodiment, leaf explants of *ex vitro* raised plants were treated with liquid basal hormone free MS medium and different osmotic agents wherein the least cumbersome and cheaper MS medium was most effective prior to bombardment with biolistic.

In yet another embodiment, leaf explants were treated with liquid basal hormone free MS medium and different ranges of osmotic agents like sucrose, myoinositol, sorbitol, mannitol alone and in combinations of mannitol and sorbitol wherein full strength hormone free basal MS medium was the most effective.

In still another embodiment, the leaf explants were treated with hormone free liquid basal MS medium and different osmotic agents for different time periods ranging from 2 to 8 hrs wherein hormone free liquid basal MS medium treatment for 4 hours was most effective.

In an embodiment, 50-70 μ g gold particle was prepared in sterile water both for direct use and storage in order to overcome the inhibitory effect of remnant glycerol during the loading of DNA onto the macro-carriers.

In yet another embodiment concentric circles of variable diameter ranging from 2.0 to 9.0cm were drawn on a transparent polythene sheet where the diameter of the outermost circle was same as that of a 9.0 cm petridish.

In an embodiment, explants were arranged with adaxial surface up on the regeneration medium for bombardment.

In another embodiment, the explants were arranged on the regeneration medium within the different concentric circles ranging from 2.0 to 5.0 cm of 9.0 cm Petri-dishes for optimization of the spreading pattern of pRT99GUS plasmid DNA coated micro-projectiles (BioRad) and using GUS assay method of Jefferson (Jefferson RA 1987, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405).

In an embodiment, gold particles ranging from 0.5-1.5 ml were sterilized by washing with 70% alcohol and sterile water for three times each.

In another embodiment, the suspension ranging from 25 to 60 μ l was dispensed in 1.5 ml Eppendorf tubes for each bombardment.

In another embodiment, 40-60 μ l of gold suspension was mixed with 5-15 μ l of different concentrations of pRT99GUS plasmid DNA (0.5-5 μ g/ μ l), 40-60 μ l of 1.5-3.5M CaCl₂ and 10-50 μ l of 0.5-2.0M spermidine free of phosphate salts.

In another embodiment, the suspension was vortexed from time to time, with spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant, washing with 70% ethanol and final suspension in 50-100 μ l of 100% ethanol.

In another embodiment, 5-15 μ l suspension of gold particles and DNA were coated on sterile macrocarriers (BioRad) with immediate vortexing.

In another embodiment, the explants were bombarded with biolistic guns like DuPont, Gene Booster and Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) but preferably Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 22 to 28 inches mercury.

In another embodiment, 360 combinations were developed comprising of: gap distances or distance between the rupture disc and the macro-carrier (1/4-3/8 inches alone and in combination), macrocarrier flight distance or the distance between macrocarrier and stopping screen (6-16 mm), and target distance or distance between the microprojectile stopping screen and target tissue (6-12 cm), for increasing the surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency

In another embodiment, 360 combinations of the above together with gold particles ranging from 0.6 to 1.6 μ m, and concentration ranging from 1, 2 and 4 μ g/ μ l of DNA were used, wherein preferably a combination of 1.0 μ m gold particles, 1100 psi burst pressure, target distance (9 cm), gap distance (3/8" + 1/4" and 1/4"), macro-carrier flight distance (16 mm) and 1 μ g/ μ l of DNA gave the maximum transformation frequency.

In another embodiment, each explant was bombarded twice by changing the direction of the Petri-plates by 180°.

In another embodiment, the bombarded explants were turned upside down on the regeneration medium with abaxial surface up.

In another embodiment, the bombarded explants preferably leaf explants were cultured in dark for two days under culture lab conditions of 25 \pm 2°C followed by culture on regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient

method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001

In another embodiment, the bombarded explants were tested for transient expression using GUS assay method of Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405 after 6 days of bombardment.

In another embodiment, the leaf derived calli were selected after every 15 days on selection medium containing kanamycin.

In another embodiment, kanamycin levels ranging from 250-1100µg/ml kanamycin was used for selection of transformants almost no chance of 'escapes'.

In another embodiment, 1.0 cm long healthy transgenic plants were grown and multiplied on kanamycin free liquid multiplication medium of Sandal I, Bhattacharya A, Ahuja P.S. An efficient liquid culture system for tea shoot proliferation Plant Cell Tissue Organ Culture 65(1): 75-80 (2001).

In another embodiment, GUS positive tissues of transgenic plants selected on 250-1100µg/ml kanamycin were characterized (molecular) using PCR and Southern Hybridization following standard methods.

In another embodiment of the present invention different explants like somatic embryos and embryogenic calli were used for bombardment with the above parameters.

In still another embodiment of the present invention leaf explants of different cultivars were used for bombardment from both *in vitro* and *ex vitro* plants.

- (i) Leaf explants were treated with liquid basal hormone free MS medium (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497; 1962) and different concentrations of osmotic agents like sucrose, myoinositol, sorbitol, mannitol alone and combinations of mannitol and sorbitol for different time periods (2- 8 hrs).
- (ii) Concentric circles were drawn on a transparent polythene sheet wherein the diameter of the outermost circle was same as that of a 9.0 cm petridish.
- (iii) Arrangement of leaf explants with adaxial surface up on the regeneration medium within the different concentric circles (2.0-5.0 cm) of 9.0 cm Petri-dishes for maximum spread of DNA coated micro-projectiles.

- (iv) Gold particles of 0.5-1.5 ml were suspended in sterile distilled water after washing with 70% alcohol and sterile water for three times each.
- (v) The suspension (25-60 μ l) was dispensed in 1.5 ml Eppendorf tubes for each bombardment.
- (vi) 50 μ l of gold suspension was mixed with 10 μ l of different concentrations of plasmid DNA (0.5-5 μ g/ μ l), 40-50 μ l of 1.5-3.5M CaCl₂ and 10-50 μ l of 0.5-2.0M spermidine free phosphate salts.
- (vii) The suspension was vortexed from time to time, with spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant and washing with 70% ethanol and final suspension in 50-100 μ l of 100% ethanol.
- (viii) 10 μ l suspension of gold particles and DNA were coated on sterile macrocarriers (BioRad) with immediate vortexing.
- (ix) The 354 combinations were developed that comprised of: (a) gap distances or distance between the rupture disc and the macro-carrier (1/4-3/8 inches alone and in combination), (b) macrocarrier flight distance or the distance between macrocarrier and stopping screen (6-16 mm), (c) target distance or distance between the microprojectile stopping screen and target tissue (6-12 cm), and burst pressure (650-1350 psi) of the rupture discs (BioRad) for increasing the surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency.
- (x) The leaf explants were bombarded with Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 25 inches Hg with 0.6-1.6 μ m gold particles, 1, 2 and 4 μ g/ μ l concentrations of DNA and the 354 combinations that were derived above.
- (xi) Each plate was twice bombarded by changing the direction of the Petri-plate by 180°C.
- (xii) The bombarded explants were turned upside down on the regeneration medium with the abaxial surface up such that the bombarded surface touches the regeneration medium for rapid healing.
- (xiii) The bombarded leaf explants were cultured in dark for two days under culture lab conditions.

- (xiv) The bombarded leaf explants were tested for transient expression using GUS assay method of Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405 in order to test the maximum spread of particle penetration.
- (xv) After two days, the bombarded leaf explants were transferred to the regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001 under normal photoperiod of culture lab conditions
- (xvi) Shoot buds were regenerated from bombarded leaf explants following the protocol of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001.
- (xvii) The putative transformants were selected after every 15 days on selection medium containing kanamycin (250-1100 µg/ml).
- (xviii) The putative transgenic shoots were grown and multiplied in liquid medium of Sandal I, Bhattacharya A, Ahuja P.S. An efficient liquid culture system for tea shoot proliferation Plant Cell Tissue Organ Culture 65(1): 75-80 (2001) Sandal I, Bhattacharya A, Ahuja P. S. 2001.
- (xix) The GUS positive tissues of transgenic plants selected on 250-1100 µg/ml kanamycin were characterized using PCR and Southern Hybridization following standard methods.

Optimization of parameters for maximum transient expression in tea leaf explants is shown in Table 1.

For successful production of transgenics through biolistic it was felt necessary to optimally combine all the different parameters that affect biolistics in order to (a) increase the surface area for maximum particle penetration (b) minimize cell damage/injury and (iii) maximize regeneration efficiency.

Use of one or two parameters depending upon the texture (hard or soft) of the tissue and the source material (genus or species) for the production of transgenics have been reported in several crops. However, the novelty of this invention is the development of a checker board of all the parameters that affect the success of biolistics that can be

universally employed. With the help of these 354 combinations of the checker board (comprising of the combinations of burst pressure of rupture disc, macrocarrier flight distance, target distance and gap distance), any transformation experiment can be successful irrespective of genus, crop or tissue. Pretreatment with osmoticum and concentration of DNA can further improve the transformation efficiency.

Pretreatment with liquid basal hormone free MS (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497; 1962) medium or 0.25M sorbitol for 4 hrs not only resulted in leathery texture of the tea leaf explants thereby enabling them to be flattened on the regeneration medium and providing a larger surface area for bombardment with minimum injury but also for healing the injury that was caused due to particle penetration. Generally, treatment with an osmotic agent prior to bombardment enhances transient expression considerably as it brings about plasmolysis of the target cells. Plasmolysis prevents the extrusion of protoplasm from cells and further reduces cell damage following particle penetration during bombardment Vain P, McMullen M.D., Finer J.J, 1993 Osmotic treatment enhances particle bombardment mediated transient and stable transformation of maize. *Plant Cell Rep* 12,84-88. Tissue pretreatment induces DNA replication resulting in a higher level of insertion of DNA into the genome (.

Path traversed by the gold particles from the stopping screen to the target tissue is generally conical in form. Therefore, overlapping of the surface area of the base of this cone with the defined concentric circles on the regeneration medium on which the explants to be bombarded are arranged for maximum that dispersion of gold particle is required. Therefore a method was devised to draw concentric circles of variable diameters (2.0-9.0 cm) on a transparent polythene sheet wherein the diameter of the outermost circle was same as that of a 9.0 cm petridish. By placing the petridish containing the target tissue on these circles and assaying them for transient expression through GUS after bombardment revealed that a concentric circle with a diameter of 2.0 cm was optimal. This is the reason why maximum particle penetration was achieved when the tea leaf explants were arranged within this area.

An increase in the burst pressure of the rupture disc, the microprojectile velocity increases tissue damage due to gas shock and high particle dispersion and results in low

transient gene expression. This was circumvented by either increasing the target distance or by keeping the tissue at a longer distance from the stopping screen for particle dispersion.

Brief description of the accompanying drawings:

In the drawing (s) accompanying this specification

Figure 1 a.b.c. represents: Leaf explant of tea plants

Figure 1 d to r represents: different transformed leaf explant showing gus expression

The following examples have been provided by way of illustration and should not be construed as limitations on the inventive concept herein.

EXAMPLE-1

Leaf explants of *in vitro* raised plants were treated with basal MS (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497; 1962) medium and different concentrations of osmotic agents like sucrose, myoinositol, sorbitol, mannitol alone and different combinations of mannitol and sorbitol for different time periods (2-8 hrs). The treated leaf explants were arranged with adaxial surface up on the regeneration medium within the different concentric circles (2.0-5.0 cm) of 9.0 cm Petri-dishes for optimization of the spreading pattern of DNA coated micro-projectiles. For bombardment gold particles in 0.5-1.5 ml were suspended in sterile distilled water after washing with 70% alcohol and sterile water for three times each and the suspension (25-60 μ l) was dispensed in 1.5 ml Eppendorf tubes. For preparation of DNA mix with 50 μ l of gold suspension, 10 μ l of different concentrations of plasmid DNA (0.5-5 μ g/ μ l), 40-50 μ l of 1.5-3.5M CaCl_2 and 10-50 μ l of 0.5-2.0M spermidine free base. The suspension was vortexed from time to time, with spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant and washing with 70% ethanol and final suspension in 50-100 μ l of 100% ethanol. 10 μ l suspension of gold particles and DNA were coated on sterile macrocarriers with immediate vortexing. The leaf explants were then bombarded with Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 25 inches Hg at 354 combinations together with 0.6-1.6 μ m gold particles, 1, 2 and 4 μ g/ μ l

concentrations of DNA and each plate was twice bombarded by changing the direction of the Petri-plates. The bombarded explants were turned upside down on the regeneration medium with abaxial surface up. The bombarded leaf explants were cultured in dark for two days under culture lab conditions followed by culture on regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001 after assaying for GUS expression following the method Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405.. Finally the leaf derived calli were selected after every 15 days on selection medium containing 250-1100µg/ml kanamycin. The GUS positive tissues of transgenic plants selected on 250-1100µg/ml kanamycin were characterized (molecular) using PCR and Southern Hybridization following standard methods.

EXAMPLE-2

Leaf explants of *ex vitro* raised plants were treated with different concentrations of osmotic agents and then transformed genetically through biolistics as stated above.

EXAMPLE-3

Leaf explants of plants of different cultivars (*Chinary, Cambod and Assamica*) were treated with different concentrations of osmotic agents and then transformed genetically through biolistics as stated above.

EXAMPLE-4

Different explants like somatic embryos, zygotic embryos and embryogenic calli were transformed genetically through biolistics as stated above.

The novelty of the method is that a checker board comprising of 354 combinations was developed which ensures the success of biolistic mediated transgenic production irrespective of the type of tissue, explant or genus.

Some of the novel features that were introduced are as follows:

1. Basal hormone free MS (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497; 1962) medium has been used for the first time instead of an osmoticum and this prevents the cumbersome and costly pretreatment steps with known osmotic agents.

2. Concentric circles of variable diameter (2.0-9.0 cm) were drawn on a transparent polythene sheet wherein the diameter of the outermost circle was same as that of a 9.0 cm petridish so that the exact area where the target tissue had to be placed would be known prior to bombardment. Moreover, this would also enable the maximization of the area for particle penetration.
3. Gold particles were suspended in water instead of glycerol for the first time in order to overcome the problems of inhibitory effects of remnant glycerol during loading of the DNA onto the macrocarriers.
4. A step for faster healing of the injuries due to particle penetration was devised by turning the leaves upside down after bombardment. This enabled the injured sites to come in contact with the medium and also resulted in faster regeneration response.
5. The responsive leaf explants i. e. after formation of the leaf callus were selected on a very high dose of kanamycin i.e. 500 or 1000 μ g/ml in order to prevent any 'escapes' and ensure a high percentage of stable transformants (95-100%). This is the first report of using kanamycin at a dose as high as 1000 μ g/ml for selection of transformants.
6. This method also ensures production of healthy transformed tea shoots.

The main advantages of the present invention are:

- 1) Elite plants to be made resistant to fungal diseases like blister blight by production of transgenics through biolistic in order to overcome the 50% crop loss.
- 2) Elite plants to be made resistant to bacterial diseases like bacterial shoot blight by production of transgenics through biolistic.
- 3) Elite plants to be made resistant to viral diseases by production of transgenic through biolistic.
- 4) Elite plants to be made resistant to tea resistant to pests like insects, thrips and mites by production of transgenics through biolistic.
- 5) Elite plants to be made resistant to herbicides like glyphosate, 2,4-D, paraquat and Diuron and pre-emergence herbicides like atrazine and oxyflurfon by production of transgenic through biolistic.

- 6) Elite plants to be made resistant to different stresses by (i) overexpressing genes encoding enzymes for increased oxygen radical scavenging (ii) for increased contents of osmolytes like mannitol, proline, fructans, glycine-betaine etc. (Bonhert et al., 1996; Hayashi et al., 1997), (iii) improving the flexibility of cell membranes and (iv) engineering the expression of stress-induced proteins like LEA proteins, dehydrins, antifreeze proteins (AFPs), heat shock proteins (HSPs) and hypoxia and anoxia reducing proteins like the VHb proteins by production of transgenics through biolistics.
- 7) Production of transgenic tea resistant to weeds like grasses, broad leaf weeds etc..
- 8) Production of transgenic tea with little or no winter dormancy.
- 9) Production of transgenic plants expressing genes like APETALA or LEAFY under specific promoter control for increased number of vegetative shoots.
- 10) Production of transgenic tea genes encoding RUBPcase under suitable promoters for enhanced photosynthetic rates.
- 11) Production of transgenic tea over expressing phytochrome gene for dense planting and good tea tables for easier plucking.
- 11) Production of tea plants with higher yield and good cup quality.
- 12) Improvement of elite plants for improved quality and yield by production of transgenics through biolistic.
- 13) Production of de-caFFEinated transgenic tea plants.
- 14) Production of transgenic tea plants with sweet tea leaves using genes like thaumatin and lectins etc.
- 15) To provide a biolistic mediated genetic transformation method for high rates of transgenic production irrespective of genus, crop, tissue, explant etc.
- 16) To overcome certain problems that are faced during some steps of biolistic mediated genetic transformation.

Table-1: Optimization of parameters for maximum transient expression in tea leaf explants

TD Pressure	3cm	6 cm	9 cm	12 cm	MFD
650psi	900, 3cm, 6mm*,	900, 6cm, 6mm *	900, 9cm, 6mm *	900, 12 cm, 6mm *	6 mm
	900, 3cm, 11mm*	900, 6cm, 11 mm *	900, 9cm, 11mm *	900, 12cm, 11mm *	11 mm
	900, 3cm, 16 mm*	900, 6cm, 16mm *	900, 9cm, 16mm *	900, 12cm, 16mm *	16 mm
900 psi	900, 3cm, 6mm,	900, 6cm, 6mm *	900, 9cm, 6mm *	900, 12 cm, 6mm *	6 mm
	900, 3cm, 11mm*	900, 6cm, 11 mm *	900, 9cm, 11mm *	900, 12cm, 11mm *	11 mm
	900, 3cm, 16 mm*	900, 6cm, 16mm *	900, 9cm, 16mm *	900, 12cm, 16mm *	16 mm
1100 psi	1100, 3cm, 6 mm*	1100, 6 cm, 6 mm*	1100,9 cm, 6 mm*	1100, 12 cm, 6 mm*	6 mm
	1100, 3cm, 11 mm*	1100, 6 cm, 11 mm*	1100,9cm, 11 mm*	1100,12 cm, 11 mm*	11 mm
	1100, 3cm, 16 mm*	1100, 6 cm, 16 mm*	1100, 9 cm, 16 mm*	1100, 12 cm, 16 mm*	16 mm

1350psi	1350, 3cm, 6 mm*	1350, 6 cm, 6 mm*	1350, 9 cm, 6 mm*	1350, 12 cm, 6 mm*	6 mm
	1350, 3cm, 11 mm*	1350, 6 cm, 11 mm*	1350, 9 cm, 11 mm*	1350, 12 cm, 11 mm*	11 mm
	1350, 3cm, 16 mm*	1350, 6 cm, 16 mm*	1350, 9 cm, 16 mm*	1350, 12 cm, 16 mm*	16 mm
1550 psi	1550, 3cm, 6 mm*	1550, 6 cm, 6 mm*	1550, 9 cm, 6 mm*	1550, 12 cm, 6 mm*	6 mm
	1550, 3cm, 11 mm*	1550, 6 cm, 11 mm*	1550, 9 cm, 11 mm*	1550, 12 cm, 11 mm*	11 mm
	1550, 3cm, 16 mm*	1550, 6 cm, 16 mm*	1550, 9 cm, 16 mm*	1550, 12 cm, 16 mm*	16 mm

- Six GAP distances ($3/8''$, $1/4''$, $1/8''$, $3/8''+1/4''$, $3/8''+1/8''$, $3/8''+1/8''+1/4''$) were tested for each of the above 27 combinations.